Flies with Parkinson's disease

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Parkinson's disease is an incurable neurodegenerative disease. Most cases of the disease are of sporadic origin, but about 10% of the cases are familial. The genes thus far identified in Parkinson's disease are well conserved. *Drosophila* is ideally suited to study the molecular neuronal cell biology of these genes and the pathogenic mutations in Parkinson's disease. Flies reproduce quickly, and their elaborate genetic tools in combination with their small size allow researchers to analyze identified cells and neurons in large numbers of animals. Furthermore, fruit flies recapitulate many of the cellular and molecular defects also seen in patients, and these defects often result in clear locomotor and behavioral phenotypes, facilitating genetic modifier screens. Hence, *Drosophila* has played a prominent role in Parkinson's disease research and has provided invaluable insight into the molecular mechanisms of this disease.

ABSTRACT

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Several million people in the world suffer from this disease and the estimated prevalence ranges from 0.01% to 1.2% of the world population. Age is the most common risk factor for PD and given that the population ages, the incidence of this disease is predicted to double by 2040 (Gustavsson et al., 2011; Kowal et al., 2013; von Campenhausen et al., 2005). Several symptomatic treatments for PD exist, but their effect is limited in time as the disease relentlessly progresses (Athauda and Foltynie, 2014; Ossig and Reichmann, 2015). Hence, a better understanding of the underlying pathology is needed to arrive at more powerful disease modifying strategies.

One of the best recognized pathological hallmarks of PD is the loss of dopaminergic neurons in the substantia nigra (Hirsch et al., 1988; Schneider and Obeso, 2014). Much of the research has therefore focused on strategies to protect these neurons from dying. Both pharmacological strategies (e.g. MPTP treatment (Blandini and Armentero, 2012; Bové and Perier, 2012)), as well as genetic strategies (e.g. POLG mutations (Dai et al., 2014; Humphrey et al., 2012; Orsucci et al., 2011)), have been exploited to kill dopaminergic neurons in model organisms, but it is questionable if the molecular mechanisms that are affected by these treatments overlap with those that are defective in PD. In fact, many of genes that are causative to PD (see below) are very broadly expressed also outside of dopaminergic neurons (d'Amora et al., 2011; Kühn et al., 2004; Mandemakers et al., 2012; Wirdefeldt and Bogdanovic, 2001). In humans, mutations in these broadly expressed genes lead to the loss of dopaminergic neurons in the brain, but the function of several other types of cells is likely compromised as well (Davie, 2008; Shulman et al., 2011). Hence, studying cellular defects caused by PD, also outside the dopaminergic system, may yield valuable insight into the molecular mechanisms that are at the basis of PD as well. It seems then that eventually, the dopaminergic neurons appear particularly vulnerable to defects in these pathways—the reasons for this are enigmatic—.

Most of the PD cases are of sporadic origin where environmental or genetic factors contribute to the development of the disease. About 10% of the cases are familial and single gene mutations are causative to the disease. Much of the research in PD has focused on these familial forms. To date 20 PD susceptibility loci have been identified, and for about half, the causative gene has been isolated (Table 1). Most of the genes involved in PD are evolutionary conserved, and they can be readily modeled in genetic model organisms, such as fruit flies, where the consequence of their loss- or gain-of-function can be studied and where genetic and pharmacological modulation of the phenotypes can be assessed. Interestingly, numerous connections between sporadic and familial forms of the disease exist: eg causative genes to PD are misregulated in sporadic forms of the disease (e.g. Parkin or LRRK2 (Chai and Lim, 2013; Lesage and Brice, 2012)), or typical protein aggregates that are often seen in sporadic and familial cases of the disease (Lewy bodies) are enriched in alpha-synuclein, a protein that is also found mutated in some familial forms (Spillantini et al., 1997).

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Table 1
Monogenetic forms of PD and its fly homolog(s). AD, autosomal dominant; AR, autosomal recessive. Adapted from Marras et al. (2012).

Symbol	Gene locus	Gene	*Drosophila* homolog	Inheritance	Disorder	Status and remarks
PARK1	4q21-22	*SNCA* (Polymeropoulos et al., 1997)	no homolog	AD	Early-onset parkinsonism	Confirmed
PARK2	6q25.2-q27	*PARK2 encoding Parkin* (Kitada et al., 1998)	*parkin*	AR	Early onset parkinsonism	Confirmed
PARK3	2p13	Unknown	–	AD	Classical parkinsonism	Unconfirmed
PARK4	4q21-q23	*SNCA*	no homolog	AD	Early-onset parkinsonism	Erroneous locus (identical to *PARK1*)
PARK5	4p13	*UCHL1*	*Uch*	AD	Classical parkinsonism	Unconfirmed
PARK6	1p35-p36	*PINK1* (Valente et al., 2004)	*pink1*	AR	Early onset parkinsonism	Confirmed
PARK7	1p36	*PARK7 encoding DJ-1* (Bonifati et al., 2003)	*Dj-1α and dj-1β*	AR	Early onset parkinsonism	Confirmed
PARK8	12q12	*LRRK2* (Paisán-Ruíz et al., 2004)	*Lrrk*	AD	Classical parkinsonism	Confirmed
PARK9	1p36	*ATP13A2* (Ramirez et al., 2006)	*CG32000*	AR	Kufor-Rakeb syndrome, a form of juvenile-onset atypical parkinsonism with dementia, spasticity and supranuclear gaze palsy	Confirmed
PARK10	1p32	Unknown	–	Risk factor	Classical parkinsonism	Confirmed susceptibility locus
PARK11	2q36-27	Unknown (maybe *GIGYF2*)	–	AD	Late onset parkinsonism	Not independently confirmed
PARK12	Xq21-q25	Unknown	–	Risk factor	Classical parkinsonism	Confirmed susceptibility locus
PARK13	2p12	*HTRA2*	*HtrA2*	AD or risk factor	Classical parkinsonism	Unconfirmed
PARK14	22q13.1	*PLA2G6* (Paisan-Ruiz et al., 2009)	*iPLA2-VIA*	AR	Early-onset dystonia-parkinsonism	Confirmed
PARK15	22q12-q13	*FBXO7* (Shojaee et al., 2008)	no homolog	AR	Early-onset parkinsonian-pyramidal syndrome	Confirmed
PARK16	1q32	Unknown (maybe RAB7L1)	–	Risk factor	Classical parkinsonism	Confirmed susceptibility locus
PARK17	16q11.2	*VPS35*	*Vps35*	AD	Classical parkinsonism	Unconfirmed
PARK18	6p21.3	*EIF4G1*	*eIF4G*	AD	Late onset parkinsonism	Unconfirmed
PARK19	1p31.3	*DNAJC6* (Edvardson et al., 2012)	*auxillin*	AR	Juvenile-onset parkinsonism	Confirmed
PARK20	21q22.11	*SYNJ1* (Krebs et al., 2013; Quadri et al., 2013)	*Synj*	AR	Early-onset parkinsonism	Confirmed

Hence, it is believed that by understanding familial PD, we will also gain insight into the sporadic forms of the disease.

PD modelling in flies

Ideal murine models for PD do not exist and this gap has maximally allowed *Drosophila* to step in as an important genetic model in the study of strategies to combat PD. While the fruit fly models also have limitations, they are small and propagate fast, allowing researchers to conduct elaborate genetic screens for modifier loci (see below); yet flies also harbour a complex nervous system with several dopaminergic neuron clusters and they are amenable to live imaging, enabling *in vivo* cell biological studies (Bier, 2005; White et al., 2010). It is likely that the neuronal connections relevant to PD in humans are likely not highly conserved in the fly brain. However, the combination of fast and elaborate genetics with in depth cell biological and molecular studies does provide a rather unique window on the mechanisms and pathways underlying PD pathogenesis *in vivo*.

The genes implicated in PD are highly conserved, and for the 14 PD genes currently identified, 12 have a fly homolog (Table 1). The two genes that are not conserved are *alpha-synuclein* (*SNCA*) and *FBXO7* and although these genes are not present in the fly genome, the human versions of these genes with or without pathogenic mutations have been overexpressed in fruit fly neurons allowing researchers to evaluate the defects that are induced and the pathways that are activated (Table 2). Such an approach may be particularly interesting for SNCA where 'gain of protein levels', caused by triplications in humans, have been shown to cause PD as well (Singleton et al., 2003). Overexpression models such as the *SNCA* models in flies are ideal to conduct biochemical and genetic screens, and this approach has been followed in *Drosophila* as well as in other model organisms. For example TRAP1 was identified in such a screen (Butler et al., 2012). TRAP1 is a mitochondrial chaperone that already had been implicated to be phosphorylated by another PD-protein Pink1 (Costa et al., 2013; Pridgeon et al., 2007; Zhang et al., 2013). Hence, such interaction screens start to elucidate common pathways that span several of the PD-relevant genes.

Similar to overexpression models, genetic analyses of almost all other PD-relevant genes has been undertaken in fruit flies. Loss-of-function models based on small gene deletions, transposon insertions or chemically induced mutations to almost all PD-relevant genes have been created. Likewise, (over)expression of wild type *Drosophila* or human versions of the genes or overexpression of pathogenic or enzymatically impaired variants have been generated. Table 2 shows an overview of the extended set of mutations and other genetic tools that have been created to date to study familial PD in flies as well as antibodies or other strategies used to localize the gene products. Fig. 1 shows the timeline of identification of PD genes versus the characterization of the corresponding fly model.

Studies of PD gene function in flies often make use of UAS-Gal4 driven overexpression of pathogenic mutations (Brand and Perrimon, 1993). Such an approach is in several instances probably warranted, but care needs to be taken as to not misinterpret overexpression artifacts, such as the induction of pathways that are normally not being activated (Liu and Lehmann, 2008). The advent of new genome editing strategies based on CRISPR/Cas9 (Bassett et al., 2013; Gratz et al., 2013; Ren et al., 2014) or TALENs (J. Liu et al., 2012; Liu et al., 2014) but also on targeted single strand annealing using MiMIC transposons, is now allowing to very efficiently and locally manipulate the fly genome as to insert tags or pathogenic mutations in PD-relevant genes (Venken et al., 2011; Vilain et al., 2014). These modified genes

Table 2
Fly PD models and tools.

Symbol	Gene	*Drosophila* homolog	Loss-of-function mutant	(Over)expression construct	(Over)expression construct with point mutation	*Drosophila* Antibody or tagged knock-in allele
PARK1	*SNCA*	no homolog	–	Yes (Chen and Feany, 2005; Chen et al., 2009; Feany and Bender, 2000; Karpinar et al., 2009; Kontopoulos et al., 2006; Trinh et al., 2008)	Yes (A30P*–A53T* (Feany and Bender, 2000), A30P*–A53T* (Karpinar et al., 2009)	–
PARK2	*PARK2 encoding Parkin*	*parkin*	Yes (Greene et al., 2003; Pesah et al., 2004; Whitworth et al., 2005)	Yes (Cha et al., 2005; Greene et al., 2003; Haywood and Staveley, 2004; Muñoz-Soriano and Paricio, 2007; Pesah et al., 2004) (also genomic construct (Pesah et al., 2004))	Yes (R42P* (Cha et al., 2005), R275W*, G328E*(Wang et al., 2007), T187A (Kim et al., 2008))	Yes (Yang et al., 2006)
PARK5	*UCHL1*	*Uch*	No	No	No	Yes (Thao et al., 2012; Tram et al., 2013)
PARK6	*PINK1*	*Pink1*	Yes (Clark et al., 2006; Park et al., 2006)	Yes (Clark et al., 2006; Kim et al., 2008; Park et al., 2006; Todd and Staveley, 2008; Yang et al., 2006)	Yes (G309D* (Wang et al., 2006), G426D* (genomic construct) (Yun et al., 2008), G426D*–L464P* (Song et al., 2013)	Yes (Park et al., 2006; Yang et al., 2006)
PARK7	*PARK7 encoding DJ-1*	*Dj-1α and dj-1β*	Yes (Lavara-Culebras and Paricio, 2007; Menzies et al., 2005; Meulener et al., 2005; Park et al., 2005)	Yes (Kim et al., 2005; Menzies et al., 2005; Meulener et al., 2005; Yang et al., 2005)	No	No
PARK8	*LRRK2*	*Lrrk*	Yes (Lee et al., 2007; Wang et al., 2008)	Yes (Dodson et al., 2012; Gehrke et al., 2010; Imai et al., 2008; Lee et al., 2007; Lin et al., 2010; Martin et al., 2014; Matta et al., 2012; Ng et al., 2009; Venderova et al., 2009) (also genomic construct (Dodson et al., 2012))	Yes (R1069G*–Y1383C*–I1915T* (Imai et al., 2008), I1122V*–Y1699C*–I2020T* (Venderova et al., 2009), Y1699C*–G2019S*–G2385R* (Ng et al., 2009),G2019S*–R1441C*–G2385R* (Lin et al., 2010), R1069G* (Gehrke et al., 2010), G1914S* (Dodson et al., 2012), G2019S* (Matta et al., 2012), G2019S* (Martin et al., 2014))	Yes (Imai et al., 2008) or use tagged knock-in allele (LRRK-HA) (Vilain et al., 2014)
PARK9	*ATP13A2*	*CG32000*	No	Yes (Schertel et al., 2013)	No	No
PARK13	*HTRA2*	*HtrA2*	Yes (Tain et al., 2009; Yacobi-Sharon et al., 2013; Yun et al., 2008)	Yes (Igaki et al., 2007; Park et al., 2006; Tain et al., 2009; Whitworth et al., 2008; Yacobi-Sharon et al., 2013; Yun et al., 2008) (also genomic construct (Tain et al., 2009; Yacobi-Sharon et al., 2013))	Yes (S236C–S266A–G363S*–S364A* (Yun et al., 2008) S266A (genomic construct) (Yacobi-Sharon et al., 2013))	Yes (Igaki et al., 2007; Khan et al., 2008)
PARK14	*PLA2G6*	*iPLA2-VIA*	Yes (Malhotra et al., 2009)	No	No	No
PARK15	*FBXO7*	no homolog	–	Yes (Burchell et al., 2013)	Yes (T22M*–R378G*–R498X* (Burchell et al., 2013))	–
PARK17	*VPS35*	*Vps35*	Yes (Belenkaya et al., 2008; Franch-Marro et al., 2008; Korolchuk et al., 2007; Port et al., 2008)	Yes (Belenkaya et al., 2008; S. Wang et al., 2014)	Yes (P316S*–D620N*–L774M* (H.-S. Wang et al., 2014))	No
PARK18	EIF4G1	*eIF4G*	No	No	No	Yes (Zapata et al., 1994)
PARK19	*DNAJC6*	*auxillin*	Yes (Eun et al., 2007; Hagedorn et al., 2006; Kandachar et al., 2008)	Yes (Eun et al., 2008; Hagedorn et al., 2006; Kandachar et al., 2008) (also genomic construct (Eun et al., 2007))	No	Yes (Eun et al., 2008)
PARK20	*SYNJ1*	*Synj*	Yes (Muñoz-Soriano and Paricio, 2007; Verstreken et al., 2003)	Yes (Dickman et al., 2006) (also genomic construct (Venken et al., 2008))	No	Yes (Verstreken et al., 2003)

* (Over)expression construct with PD-associated mutation.

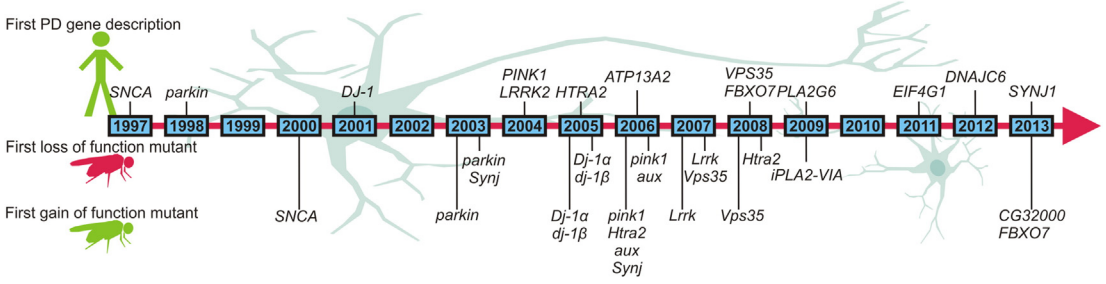

Fig. 1. Timeline illustrating the generation of fly PD models, indicating when the first human PD genes where described above the timeline and the generation of the first *Drosophila* loss- and gain-of-function mutant below the timeline.

are now expressed at endogenous levels. Such technology was already used to tag the endogenous fly *LRRK* gene with HA for endogenous protein localization (Vilain et al., 2014) and such approaches will become more and more mainstream as they more closely mimic gene dosage in patients.

Flies are well-suited for "*in vivo*" cell biological studies related to pathogenic pathways in PD because such studies can be readily combined with functional *in vivo* studies and many genetically identical animals can be analyzed. For example, mitochondrial activity in thoracic and larval muscle cells can be effectively assessed using fluorescent and biochemical assays and the defects observed can be paired with motor defects such as negative geotaxis or the ability of the flies to fly (Ali et al., 2011). Similarly, it is possible to use cell type specific expression of transgenes to assess neuronal cell biology in combination with electrophysiology and electron microscopy at neuromuscular junctions or with confocal imaging in dopaminergic neurons in the fly brain (Brand and Perrimon, 1993; Cao et al., 2013; Engel, 2008; Ramachandran and Budnik, 2010; White et al., 2010; Zhang and Stewart, 2010). Flies can also be aged and the defects that are observed in PD models can be assessed over time. Flies live a relatively short life (<80 days) which facilitates analyses of nervous system phenotypes across the complete lifespan. An important drawback here is that this time frame may be too short for dysfunctional proteins or protein aggregates to accumulate, thus not recapitulating this aspect of pathology. Nonetheless, the expanded repertoire of functional and cell biological assays in combination with the powerful genetic toolbox is exceptional, providing a unique inroad into studying the mechanisms of PD.

Pathways in PD

Familial PD is caused by mutations in different loci, and the identification of the genes is not revealing one specific pathway that is affected. Similarly, the sporadic factors that are causative to the disease may be diverse as well. It is therefore critical to study the different genetic causes of PD as to assess overlap in pathways but also the differences. Such studies will not only help to identify more effective treatment options but they will also aid in stratifying the PD patient population.

Misregulation of microRNAs

One of the pathways that is emerging in PD is misregulation of microRNAs (miRNA) and work in fruit flies suggests LRRK2 may be a central player in this process. Fly LRRK binds to Argonaute-1, a central component of the RNA-induced silencing complex (RISC). Further work has shown that pathogenic LRRK2 inhibits let-7 (a miRNA) and miR-184 and this results in the upregulation of two transcription factors E2F1 and DP; a defect connected to locomotor activity defects in flies (Gehrke et al., 2010). Interestingly, the expression of SNCA mRNA is negatively regulated by at least two microRNAs, miR-7 and miR-153 (Doxakis, 2010; Junn et al., 2009) and DJ1 and Parkin expression has also been suggested to be connected to reduced miR-34b/c expression in late stage PD brain samples (Miñones-Moyano et al., 2011). While much more work is needed to elucidate which and how miRNAs are controlling PD-gene expression, the discovery that LRRK2 may more directly affect the expression and function of miRNAs themselves may suggest a more direct connection between PD and miRNA function (Ma et al., 2013; Mouradian, 2012).

Vesicle trafficking defects

Misregulation of the expression of PD genes may have profound effects on cellular and neuronal function and work in flies has provided important insight into these mechanisms. In recent years a role for vesicle trafficking has emerged and several of the genes involved in PD have been suggested to play an active role in the process. Alpha-synuclein, LRRK2, Vps35, Synaptojanin, Parkin and DNAJC6/auxillin are directly connected to the process and several of these proteins have been extensively studied in fruit flies (Dodson et al., 2014; Korolchuk et al., 2007; Linhart et al., 2014; MacLeod et al., 2013). Interestingly, work in *Drosophila* is also helping to start elucidating the connections between these different players; e.g. between *SNCA* and *Vps35* (Miura et al., 2014) or between *LRRK2* and *Synaptojanin* (Matta et al., 2012; Verstreken et al., 2003). Indeed, fly and human LRRK2 can phosphorylate one of the central players in synaptic vesicle endocytosis: Endophilin A, and Endophilin A very tightly binds to Synaptojanin (Ringstad et al., 1997; Verstreken et al., 2002, 2003). Phosphorylation of Endophilin A affects its membrane binding and membrane remodeling properties affecting synaptic vesicle recycling (Ambroso et al., 2014; Matta et al., 2012). While fly Endophilin A is phosphorylated at Serine75, a residue central in the regulation of Endophilin A-membrane interactions, mammalian Endophilin A was recently shown to be phosphorylated at Serine 75 and at Threonine 73 that are located very closely to one another (Arranz et al., 2014; Matta et al., 2012). It is in this context interesting to note that Endophilin A is also ubiquitinated by Parkin and Parkin levels are strongly upregulated in *endophilin A* mutant mice (Cao et al., 2014; Trempe et al., 2009). Hence, discoveries in *Drosophila* are adding to the network that is emerging around Endophilin A, but how the function of Endophilin A is connected to the pathogenesis in PD remains to be elucidated.

Providing additional links between PD and synaptic endocytosis, work in flies, mice, nematodes and zebrafish indicate the central role of Synaptojanin in synaptic vesicle uncoating following new vesicle formation (Cremona et al., 1999; Harris et al., 2000; Van Epps et al., 2004; Verstreken et al., 2003). Synaptojanin is a phosphoinositide phosphatase involved in the dephosphorylation of $PI(4,5)P_2$ (Guo et al., 1999; McPherson et al., 1996), a lipid required for endocytosis (Jost et al., 1998; Koch and Holt, 2012). The work indicates that the absence of Synaptojanin function results in strong defects in the reformation of a synaptic vesicle pool following stimulation because of defects in protein uncoating from nascent synaptic vesicles. Interestingly, DNAJC6/auxillin has been implicated in exactly this same process as well (Ahle and Ungewickell, 1990; Hirst et al., 2008; Scheele et al., 2001; Ungewickell et al., 1995; Yim et al., 2010), suggesting that this function is somehow connected to PD-relevant pathways. However, the links between synaptic vesicle uncoating and neurodegeneration are yet to be elucidated.

These findings in fruit flies, but also the work in many other systems strongly implicate a group of PD-relevant proteins rather directly in (synaptic) vesicle trafficking steps (Braschi et al., 2010; Cirnaru et al., 2014; Gitler et al., 2008; McLean et al., 2000; Piccoli et al., 2011; Soper et al., 2011; Vargas et al., 2014; Yun et al., 2013). This work then opens the question as to how defects in vesicle trafficking can be connected to PD? Vesicle trafficking is closely connected to autophagy where pre-autophagic membrane vesicles fuse to create autophagosomes that engulf debris and destine it for degradation (Ge et al., 2014; Yamamoto et al., 2012). It is conceivable that defects in this turnover pathway may result in cellular stress as a result of defects in protein turn over. However, how defects in autophagy cause cellular dysfunction in specific cell types remains to be elucidated. Vesicle trafficking defects may also cause altered localization of membrane bound receptors thus affecting signaling pathways. At the synapse, defects in vesicle recycling will also lead to defects in neurotransmitter release, thus causing altered information transfer in neuronal circuits. In dopaminergic neurons, slower vesicle recycling would in theory result in less dopamine uptake into synaptic vesicles and increased cytoplasmic dopamine levels. Dopamine is toxic and creates reactive oxygen species (ROS) (Lotharius and O'Malley, 2000) and removing it from the cytoplasm may be protective. In line with this idea, over expression of the vesicular dopamine transporter is protective in PD-fly models (Lawal et al., 2010). Hence, several models that connect defects in vesicle trafficking to cellular survival are

emerging (Esposito et al., 2012), but additional work is needed to link this process to pathology.

Mitochondrial dysfunction

While vesicle trafficking defects in PD are emerging in recent years, mitochondrial dysfunction in the disease is recognized since long and both sporadic and genetic forms of the disease have been connected to these organelles. Post mortem brain samples from PD patients often show mitochondrial defects (Arthur et al., 2009; Keeney et al., 2006; Naydenov et al., 2010), and pesticides or mitochondrial toxins have been connected to PD-like symptoms in humans and laboratory animals (Betarbet et al., 2000; Blandini and Armentero, 2012; Bové and Perier, 2012; Langston et al., 1983). In addition, several of the PD-proteins have also been shown to affect mitochondria, including DJ-1, Pink1 and Parkin. Interestingly, PD-causing mutations in all these genes are recessive and loss-of-function animals are thus good systems to model these types of PD. Also here, fruit fly research has made important contributions. For example DJ-1 mutant *Drosophila* show compromised mitochondrial function with age (Hao et al., 2010), and these defects are also seen in DJ-1 mutant mice (Irrcher et al., 2010). Interestingly, also *pink1* and *parkin* mutant flies show mitochondrial defects that overlap with the defects seen in the DJ-1 mutants (Hao et al., 2010). Further work is now needed to connect the role of DJ-1 to the functions of Pink1 and Parkin in the regulation of mitochondrial function. Furthermore, insight into how such mitochondrial defects would then lead to neuronal loss, in particular dopaminergic neuron loss is also awaited.

One of the significant contributions fruit fly research has made to our understanding of PD relevant pathways was the discovery of mitochondrial defects in genetic models of familial cases of PD. *Parkin* mutant flies, but also *pink1* mutant flies show abnormally positioned wings, dented thoraces and locomotor defects (Clark et al., 2006; Greene et al., 2003; Haywood and Staveley, 2004; Park et al., 2006; Pesah et al., 2004; Yang et al., 2006). Examination of the flight muscles showed disorganized muscle fibers with enlarged mitochondria, explaining the thoracic defects. Although the mitochondrial defects in *parkin* or *pink1* mutants are most obvious in muscle cells that are energy demanding, other cell types, including the dopaminergic neurons in the fly brain, also appear to suffer from mitochondrial defects, suggesting loss of these genes results in systemic mitochondrial defects, but not all tissues suffer to a similar extend (Clark et al., 2006; Greene et al., 2003; Haywood and Staveley, 2004; Park et al., 2006; Pesah et al., 2004; Yang et al., 2006). These early discoveries have then paved the way to much more elaborate investigations that have really gone to the core of the mitochondrial defects. In parallel, addressing these fundamental questions has also provided many exciting avenues in potential therapeutic strategies that are now waiting to be translated.

The similarity between the phenotypes in *parkin* and *pink1* mutant fruit flies is very striking, and also in humans, PD-patients suffering from mutations in the orthologous genes show very similar early onset and slow disease progression phenotypes (Lohmann et al., 2003). These strong overlaps in phenotypes suggested that these genes may act, at least in part, together to protect mitochondria from being damaged. This idea was first tested in fruit flies by showing that expression of wild type Parkin in a *pink1* mutant partially alleviates the *pink1*-associated phenotypes. Conversely, expression of Pink1 in a *parkin* mutant does not rescue *parkin*-associated phenotypes, suggesting that Parkin acts in this role downstream of Pink1 (Clark et al., 2006; Park et al., 2006). These observations are interesting in light of an emerging idea that Parkin is needed to the degradation of sick mitochondria by autophagy (mitophagy) and proteasomal degradation of mitochondrial components (Kawajiri et al., 2010; Narendra et al., 2009; Vincow et al., 2013; Yamamoto et al., 2005).

While many questions still remain, work in flies has helped to elucidate important aspects of this mitochondrial quality control pathway. Mitochondria undergo constant fusion and fission, and it is believed that this process is needed to exchange mitochondrial material and maintain a healthy mitochondrial pool. It is also thought that mitochondrial fission precedes mitochondrial autophagy as to separate the dysfunctional mitochondrial pieces from the healthy ones. In line with this idea, early work in flies indicated that increased fission or decreased fusion restores many of the mitochondrial morphological defects seen in *pink1* and *parkin* mutants (Deng et al., 2008; Poole et al., 2008; Yang et al., 2008). One explanation of these observations is that indeed more fission aids the removal of these dysfunctional mitochondria.

Parallel findings in mammalian cell culture and *Drosophila* S2 cells suggest that under conditions where mitochondria are poisoned, Pink1 is stabilized on the mitochondrial outer membrane as to recruit Parkin (Korolchuk et al., 2007; D. P. Narendra et al., 2010; Ziviani et al., 2010). The stabilization of Pink1 then appears to trigger a series of events that results in the degradation of these sick mitochondria. Pink1 is a kinase and when Pink1 is stabilized on dysfunctional mitochondria, it phosphorylates Miro, a protein that mediates kinesin dependent mitochondrial transport (S. Liu et al., 2012; Tsai et al., 2014; Wang et al., 2011). Further work indicates that phosphorylated Miro is degraded by Parkin, an E3 ubiquitin ligase that is activated by the E2 conjugase Ube2A/Rad6 in mammalian cells and in flies (Haddad et al., 2013). Hence, the work conducted both in mammalian neurons and in flies, suggests that dysfunctional mitochondria fail to be transported in the cell. Stabilized Pink1 also targets other proteins: it recruits and phosphorylates Parkin (Shiba-Fukushima et al., 2012) and ubiquitin (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014; Shiba-Fukushima et al., 2014), and Parkin then ubiquitinates mitochondrial targets that tag the organelle for degradation. The evidence indicates this pathway is implicated in mitochondrial quality control, but the targets ubiquitinated by Parkin including Mitofusin (Poole et al., 2010; Ziviani et al., 2010) and VDAC (Geisler et al., 2010; D. Narendra et al., 2010) also regulate mitochondrial-endoplasmatic reticulum contacts (Rowland and Voeltz, 2012), further causing cellular dysfunction.

While this model is appealing, there are however also still a number of important but unanswered questions. For example, increased mitochondrial fission does not rescue the functional defects that are observed in *pink1* mutant mitochondria, indicating that at least Pink1, also acts in other pathways of mitochondrial activity (Liu et al., 2011; Vilain et al., 2012). In addition, it has proven challenging to observe mitochondrial autophagy *in vivo* in neurons under non-artificial conditions, making it difficult to assess the *in vivo* importance of the process also during ageing. The recent development of killer red mitochondria may help to elucidate the process further. Here, killer red that is expressed in mitochondria creates ROS when excited and these ROS result in damage and the recruitment of parkin to these mitochondria (Ashrafi et al., 2014). Finally, if Pink1 is required to recruit Parkin to mitochondria to promote mitophagy, how is the overexpression of Parkin able to rescue the *pink1* mutant phenotypes? Possibly alternative recruitment mechanisms or alternative autophagic strategies may exist and become upregulated, but these mechanisms, if they are relevant, remain to be studied in this context.

In healthy mitochondria much of the Pink1 protein is proteolytically cleaved by PARL (Whitworth et al., 2008). However, the remaining Pink1 appears to fulfill an important function. Work in mouse *Pink1* knock out cells and in *pink1* fly mutants indicates enzymatic defects in the electron transport chain (ETC) in the inner mitochondrial membrane (Gautier et al., 2008; Liu et al., 2011; Morais et al., 2009). The ETC transports electrons down their electrochemical gradient to establish a proton gradient over the inner mitochondrial membrane and this gradient is used by the mitochondrial ATPase to produce ATP. In *pink1* mutant flies and cells, the enzymatic defects at the level of complex I result in a less negative

mitochondrial membrane potential and less ATP production. Proteomic studies combined with *in vivo* rescue experiments in flies, mouse and patient derived cells subsequently provided evidence that *pink1* deficiency results in defects to couple electron transport between complex I and ubiquinone (Morais et al., 2014). Both in flies and mammalian cells, the process of electron transfer at this level is facilitated by the phosphorylation of a specific complex I subunit NDUFA10, suggesting that therapeutic strategies that target this site may promote mitochondrial function (Morais et al., 2014; Pogson et al., 2014). Further supporting this model, in flies, the mitochondrial defects in *pink1* mutants are rescued by expression of a yeast protein, NDI1 that is able to bypass electron transport in complex I by feeding electrons straight into complex III (Pogson et al., 2014; Vilain et al., 2012). Hence, a model emerges where Pink1 double-times between trying to maintaining high mitochondrial function and, when that fails, mediating Parkin dependent events.

Using flies to find new treatments for PD

Future work can now gear towards using this knowledge to start developing therapeutic strategies, also here, fruit flies have proven their value. Pink1 has been routinely used as a model, most likely because of pragmatic reasons: in flies *pink1* is on the X-chromosome, facilitating genetic screens, but also because Pink1 has been intensely characterized and studied. It is likely that other PD models will follow in the future as well. Nonetheless, genetic screens in fruit flies and gene expression profiling in *pink1* mutants have been very successful and they also further point to a mitochondrial connection. Indeed, the loss of Pink1 function activates a metabolic pathway that results in increased levels of nucleotides that appear to promote mitochondrial biogenesis, thereby suppressing the mitochondrial defects. Moreover, it was shown that enhancing nucleotide metabolism either genetically or using pharmacology is able to protect against mitochondrial dysfunction in *pink1* mutants (Tufi et al., 2014). Further clinical studies will now need to be undertaken to assess the applicability of this approach in therapy.

An effective strategy to identify suppressors of the *pink1* associated defects has been the use of genetic screening in fruit flies. *Pink1* mutant flies do not fly well, likely because of mitochondrial defects in the muscle cells. Dominant suppressor screens then search for second site mutations (other than the *pink1* mutation) that alleviate the defect in flight. This is a fast and effective way to identify suppressor loci, but the disadvantage is that such screens aim to identify factors that suppress the mitochondrial defects in *pink1* mutant muscle cells, not neurons. Hence, neuron specific factors may be missed. Nonetheless, such screening approaches have identified numerous modifier loci (Esposito et al., 2013; Fernandes and Rao, 2011; S. Liu et al., 2012; Pogson et al., 2014; Thomas et al., 2014; Vos et al., 2012; Wu et al., 2013). For example, an enzyme that is involved in the production of vitamin K2 is a dosage sensitive modifier of the *pink1* defects in flies. While vitamin K2 is best known for its role in blood coagulation, subsequent work indicated that vitamin K2 is also abundantly present in mitochondria, and that the compound acts as an alternative electron carrier molecule. In *pink1* mutants, the action of vitamin K2 overcomes the ETC defect and results again in effective ATP production (Vos et al., 2012). Similarly, other strategies that improve mitochondrial function, including those that scavenge the ROS produced by the *pink1* mutant mitochondria are protective. For example GST or removing Aconitase, a major mediator of ROS-induced toxicity in mitochondria are protective (Esposito et al., 2013; Kim and Yim, 2013; Whitworth et al., 2005). In addition, expression of Ret, TNF receptor-associated protein 1 (TRAP1) and near-infrared 808 nm light all turned out to improve mitochondrial function in *Drosophila pink1* mutants thereby rescuing the cellular and behavioral defects in these animals (Klein et al., 2014; Vos et al., 2013; Zhang et al., 2013). These strategies are thus paving the way to a new generation of therapeutic strategies in Pink1-dependent strategies.

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